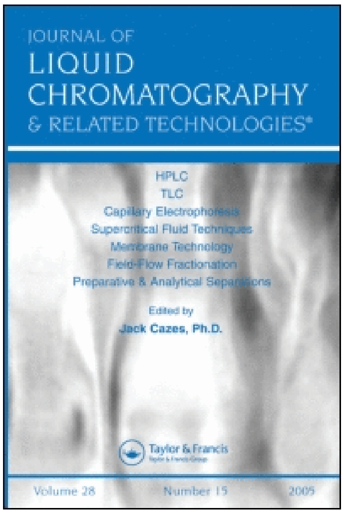

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Multidimensional Liquid Chromatographic Separations Applied to the Analysis of Food Samples

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Abstract: Nowadays, multidimensional techniques occupy an expanding niche in separation science, being strong evidence that one-dimensional systems are by themselves inadequate, especially when challenged with highly complex samples. No exceptions are foodstuffs: analytical multidimensional methods not only allow the qualitative and quantitative determination of the main compounds, but can also be selective and sensitive enough to permit the determination of minor components. The present review gives an overview of the application of three main types of multidimensional separations, off-line, on-line/heart-cutting, and comprehensive liquid chromatography, for the analysis of food and beverages. The principles, advantages, and disadvantages of each approach are briefly described.

Keywords: Food analysis, LC × LC, Liquid chromatography, Multidimensional separations, Off-line LC-LC, On-line LC-LC

INTRODUCTION

The concept of food composition can and should be inclusive of all intrinsic, deliberately added, and incidental components,[1] including

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organic (carbohydrates, proteins, lipids, vitamins) and inorganic nutrients (water, oxygen, minerals), as well as bioactive non-nutrients, anti-nutrients, toxicants, and contaminants. Food chemistry is involved in elucidating both the composition of the raw materials and final products, as well as the changes which occur in food during production, storage, and processing. The analysis of food products may be directed to the assessment of food quality and authenticity, the control of a technological process, the determination of nutritional values, and the detection of molecules with a possible beneficial or a toxic effect on human health.

Analytical methods allow the qualitative and quantitative determination of the main components of food samples, but can also be selective and sensitive enough to permit the determination of minor components. Consequently, a major objective in food chemistry concerns the continuous improvement and development of analytical techniques.

High performance liquid chromatography (HPLC) has acquired a role of great importance in food analysis, as demonstrated by the wide variety of applications reported. Whilst single column chromatographic processes have been widely applied for real-world sample profile elucidation, in this area, providing high degrees of resolving power, they frequently approach or exceed their limits, especially when applied to matrices presenting a high level of complexity. As a consequence, despite a careful method optimization procedure, conventional HPLC may sometimes be inadequate. Moreover, peak overlapping may occur, even in the case of relatively simple samples, containing components with similar properties. It is well known that, even in samples of low complexity, peaks are randomly distributed and, thus, high plate number values are required for total peak resolution.[2,3] Davis and Giddings have shown that, if the number of components exceeds 37% of the peak capacity, peak resolution is statistically compromised.[2] A possible solution to circumvent this problem can be the use of multidimensional (MD) systems, where the dimensions are based on different separation mechanisms.[4,5]

Multidimensional liquid chromatography (MD LC) is a technique in which a sample is first subjected to separation via one method and then the resolved components are further separated by at least one additional method. Giddings[6] designated the conditions for an MD system:

1. the separation may be defined *multidimensional*, if components of the sample are subjected to two or more independent separation steps, in which their displacements depend on different factors;
2. whenever the two components are adequately resolved in one separation step, they must remain separated throughout the entire separation process.

Although there is no inherent restriction to the number of independent separation methods used in an MD separation, practical constraints have limited the vast majority of the MD separations reported to date to two dimensions.

On historical terms, the use of coupled columns in HPLC has attracted strong interest since its introduction more than 30 years ago.[7] Development in this area, facilitated by the HPLC apparatus technical progress and stimulated by the high demands required for analytical methods, has been continuous, leading to comprehensive two-dimensional liquid chromatography (LC × LC) systems, (introduced for the first time in 1978 by Erni and Frei),[8] where the whole sample is analyzed on-line by the two LC systems.

MD separation systems can be used either in the target[9,10] or profiling mode.[11–13] Although the common coupled-column LC system (LC-LC) can be successfully used to isolate and resolve selected regions of the sample, the comprehensive two-dimensional (2D) mode is capable of giving a broad overview of the sample.

The potential of MD techniques can be furtherly increased by means of on-line coupling of different detection systems, i.e., photo diode array (PDA) and mass spectrometry (MS), thus greatly enhancing the identification power.

The present review provides a snapshot on the application of multidimensional LC to the analysis of food and beverages. It must be noted that the combinations of HPLC with other chromatographic methods are not considered. Those regarding comprehensive chromatographic techniques in food analysis have been recently reviewed.[14,15] The principles, advantages, and disadvantages of different types of MD LC techniques are only briefly described, considering that deeper information, regarding technical aspects, can be found elsewhere (e.g.[4,5,16,17]). The applications part of the review is divided into three main sections, devoted to off-line LC-LC, on-line LC-LC, and comprehensive LC techniques.

GENERAL ASPECTS

Two-dimensional HPLC can be performed either off-line or on-line. Theoretically, many combinations may be employed, generating increased peak capacity, selectivity, and resolution, especially in the comprehensive LC mode. However, the combination of certain LC modes can present a series of difficulties, if not impossibilities, such as, for example, mobile phase immiscibility or the incompatibility between the mobile phase of the first and the stationary phase of the second dimension.

Off-Line MD LC

In off-line HPLC, the fractions eluted from the primary column are collected manually or by a fraction collector, concentrated if necessary, and then re-injected onto a second column. The benefits of this approach are as follows:

1. it is easy to carry out, as there is no need for special interfaces or switching valves;
2. both separations can be performed using only one HPLC system by changing the mobile phases and/or columns;
3. there are no problems related with incompatibility of the solvents of the two dimensions; thus, a great variety of different HPLC modes can be coupled;
4. the detection of trace components is possible by collecting and concentrating large volumes of the 1D effluent;
5. the sample concentration of both separative dimensions can be easily regulated;
6. the two dimensions can be completely independent; there is no need to adapt the flow-rates or analysis times.

The disadvantages of the off-line approach are:

1. it is labour-intensive;
2. it is time-consuming;
3. it is difficult to automate;
4. sample loss or degradation can occur during handling;
5. possibility of sample contamination or artefact formation during long treatment;
6. sample recovery is often low.

The off-line approach is quite easy, because both analytical dimensions can be considered and optimised as two independent methods. This technique is mainly used when only parts of the first separation require a secondary separation, or as a pre-separation step.

On-Line MD LC

In an on-line HPLC system, the two columns are connected by means of a special interface, usually a switching valve, which allows the transfer of the fractions of the first column effluent onto the second column. Very often, only certain parts of the sample are subjected to the two-dimensional separation and only a certain number of multi-component effluent fractions are re-injected from a primary to a secondary column. In this case the term “heart-cutting” is used.

The on-line approach is characterized by the following advantages if compared to the off-line mode:

1. it is quite easy to automate (thus offering improved sample throughput);
2. minimized sample loss or contamination;
3. reduced analysis time;
4. more reproducible;
5. shorter sample-treatment time (important while analysing unstable compounds).

However, this technique presents a number of challenges:

1. it is more difficult to operate: automated or semi-automated systems are needed;
2. more thought must be given to method and instrument design (above all when the goal is automation);
3. more instrumentation is needed (most on-line LC-LC techniques require the use of multiple LC pumps);
4. need for specific interfaces;
5. incompatibility of different mobile phases: immiscibility, precipitation of buffer salts, incompatibility between the mobile phase from one column and the stationary phase of the other;
6. separation obtained in the first column can be partly reduced in the second;
7. different separation modes are more difficult to be coupled.

On-line LC-LC is a powerful technique, frequently used also for sample clean-up, because it can be used for efficient matrix elimination, and, hence, is characterized by less time-consuming sample treatment steps than in off-line approaches.

Using MD LC in the column switching mode, highly selective separations are possible through the choice of distinct transfer techniques and switching functions, as well as changing the chromatographic modes of separation during the overall separation process. The goals in applying column switching can be to increase resolution, selectivity, and sensitivity, to enrich trace amounts of the sample, to protect sensitive detectors or to speed up the column stabilisation. However, prior knowledge of the sample composition is always beneficial when using heart-cutting MD LC.

Comprehensive Two-Dimensional HPLC

A typical comprehensive two-dimensional HPLC separation is attained through the connection of two columns by means of an interface (usually a high pressure switching valve), which continuously entraps specific quantities of first dimension eluate and directs them onto a secondary column. The type of interface depends on the method used, although multiport valve arrangements have been the most frequently used.

It has been stated that a LC × LC separation should possess the following features (in addition to those relative to general MD separation, described in the previous paragraph):[18,19]

- Equal percentages (either 100% or lower) of all sample components pass through both columns and eventually reach the detector.
- The elution profiles from both dimensions are preserved.

The advantages and disadvantages of the LC × LC approach coincide to a great extent to those of the on-line approach. In addition, the following advantages can be emphasized:

1. higher resolution (because of the unlikelihood of two components having the same retention times in both dimensions);
2. operator is less involved in the analysis process;
3. greater amount of sample information can be obtained with a single analysis;
4. great potential for the identification of "unknowns" (due to the formation of group-type patterns on the two-dimensional plane);

as well as disadvantages:

1. need for specific interfaces and software (for instrument control and, above all, data visualization);
2. the two dimensions have to be optimized in parallel, one is dependent on the other.

In the case of complex samples containing components with a certain degree of structural similarities or regular differences (for example, molar mass or end-group differences in polymers, different number of repeat units in homologues or homo-oligomers, equal structural units at different positions in the molecules in positional isomers, different numbers and annelation of aromatic rings in polyaromatic hydrocarbons, or different number and polarities of functional groups), regular variation of the structures of the compounds often leads to regularity in a chromatogram and the properties that cause this regularity or ordering can be exploited in a 2D system to interpret an orderly MD chromatogram.[20–22]

However, comprehensive HPLC techniques are complicated by the operational aspects of switching effectively from one operation step to another, by data acquisition and interpretation issues. Therefore, careful method optimization and several related practical aspects should be considered.

MULTIDIMENSIONAL HPLC APPLICATIONS

Off-line MD LC Applied for the Evaluation of Food and Beverages Composition

The oldest and most common form of multidimensional LC is the off-line approach. This can be called as “cross-fractionation” or “heart-cut” method, because, generally, only some fractions from the first-dimension effluent are collected and re-injected into a second liquid-chromatographic system. The resulting data are two or more linear chromatograms. This technique requires knowledge of the retention of specific sample components, before the fractionation can take place and it is commonly used for the separation of (a) specific component(s) in a sample.

Numerous couplings of various LC modes for the off-line LC-LC separations have been used, both for the analyses of food samples, as well as for the purification and pre-separation of the sample prior to analytical separation using other techniques. Certainly, it is not possible to report all the vast number of applications of off-line MD LC, but a selection has been made in order to demonstrate the possibilities and some examples related to the use of off-line LC-LC. Considering that the studies on the separation of biological macromolecules (peptides, proteins) and lipids (triacylglycerols in particular) represent the highest number of papers found in the literature, separate paragraphs will be dedicated to the research made on these compounds. In addition, a brief overview on the use of this technique for the analysis of several other compounds in different food matrices will be given.

Separation of Triacylglycerols (TAGs)

An application area of particular interest of multidimensional chromatography is that of edible oil and fat analysis. The main constituents of an edible fat/oil are the triacylglycerols (TAGs), which consist of a glycerol backbone, where each of the three OH groups is esterified with a fatty acid (FA).

It has to be noted that there are two main methods for the HPLC determination of TAGs. The most widespread method is non-aqueous reversed phase (NARP) HPLC. By using this approach, the elution order is dependent on increasing partition number (PN=CN-2DB).[23] Silver-ion (SI, Ag or Ag^+) HPLC is also widely used in TAG separations and the elution sequence is connected to an increasing degree of unsaturation and to position/configuration of the double bonds within each FA. The separation mechanism is based on specific silver-ion/double bond interactions.[24]

Various detection systems have been employed in HPLC separations of TAGs, such as evaporative light-scattering (ELSD)[25,26] and electrospray ionisation mass spectrometry (ESI-MS),[27–29] even if atmospheric pressure chemical ionization (APCI) MS has gained quite a large employment in HPLC triacylglycerol analysis, as it can provide valuable information on FA structures and position.[30–33]

The determination of TAG compositions in food matrices is rather a challenging and complicated task, due to the complex nature of these samples, since the wide variety of fatty acids occurring in natural lipids often creates an intricate pattern of compounds. Consequently, more effort has been applied to the separation and determination of TAGs than to any other single lipid class.

It has been demonstrated that coupling the NARP-HPLC in the first separation and Ag-HPLC in the secondary separation can provide great amount of information about the molecular species of TAGs in several samples, e.g., partially hydrogenated palm and soybean oil,[34] rice oil,[35] donkey milk,[36] and animal fats.[37] In all these cases, the preparative separation (according to PN numbers) into fractions is performed using a RP C18 column. The secondary silver-ion step separates the fractions using either an Ag-column, as reported by Dugo et al.[35–37] or RP column with mobile phase containing $AgNO_3$, as reported by Petersson et al.[34] Petersson et al. investigated the mobile phase composition containing silver ions and achieved the separation and quantification (% of total TAGs) of 18 TAGs in partially hydrogenated soybean and palm oil, including TAG geometrical isomers as well as *cis*- and *trans*- isomers of linoleic acid. However, the employment of this method did not allow the separation of positional isomers (e.g. POP/PPO). In addition, the FA composition of both oils was determined by GC.

The separation of TAG positional isomers can be achieved using a silver ion column in the second dimension. For example, Dugo et al. investigated the rice oil TAG profile including positional isomers.[35] The confirmation of the presence of different isomers was carried out through the determination of diglyceride ions ratio derived from mass spectrometric detection (with APCI interface). By combining the MD HPLC with APCI-MS detection, the isomers of 11 TAGs (with relative abundance of different isomers reported) were detected. A similar NARP-Ag/APCI-MS method was used for the evaluation of the TAG fraction in animal fats, lard, and tallow[37] and donkey milk fat.[36] The latter work describes the evaluation of a truly complex sample, where more than 43 TAGs were detected and quantified (relative % of total TAGs), while the comparison of the two animal fats (lard and tallow) was performed in the former work. This method was suitable also for the quality control, allowing the determination of small amounts of tallow in lard (by exploiting the positional isomers ratios of different TAGs present in these fats).

Other authors describe the opposite situation, where the silver ion chromatography is used in the first and NARP in the second separation step, e.g., for the fractionation of TAGs in herring oil,[38] and butterfat.[39–41] Light scattering and mass spectrometric detection have been used.

The TAGs of North Atlantic herring oil were separated by preparative HPLC on a column of silica gel from other classes of lipids, followed by the sequential combination of silver ion and reversed phase HPLC. 11 fractions were obtained with the separation using silver-ion chromatography, which was subsequently divided into 130 fractions by reversed phase HPLC for the fatty acid analysis. The latter was executed by gas chromatography, determining the FA methyl esters (FAMEs). Figure 1 shows an example of the separation of total TAGs in herring oil (Figure 1a) and separation of TAGs of 4 silver-ion fractions (Figure 1b-e). The described work showed that the sequential analyses using complementary techniques and mass spectrometric detection enables to obtain a considerable amount of information regarding the composition of molecular species of TAGs, but the identification of some compounds can be complicated or impossible.

A similar Ag-RP HPLC method was used for determination of the TAG composition of two solid and two liquid fractions of butterfat obtained by the Tirtiaux melt fractionation process.[39] The TAGs of each of the four fractions and butterfat were purified, separated by Ag-HPLC into six subfractions; each was separated by RP-HPLC and analyzed by GC for the composition of FAs (in terms of FAMEs). The study demonstrates that, although the interpretation of the results can be complicated (due to the incomplete separation of liquid phases from the solid ones), the sequential combination of the two HPLC methods used provides more information and better understanding of the chemical selectivity of the Tirtiaux process than these methods used in parallel.

The same group continued their work by analysing the butterfat containing configurational isomers of monoenoic fatty acyl residues.[40,41] The Ag-RP HPLC method used in these studies is the same as in the first work;[39] in addition, the MS and tandem MS (MS/MS) were used for structural elucidation of TAGs. These works show that the combined use of multidimensional HPLC, MS and GC allows the elucidation of

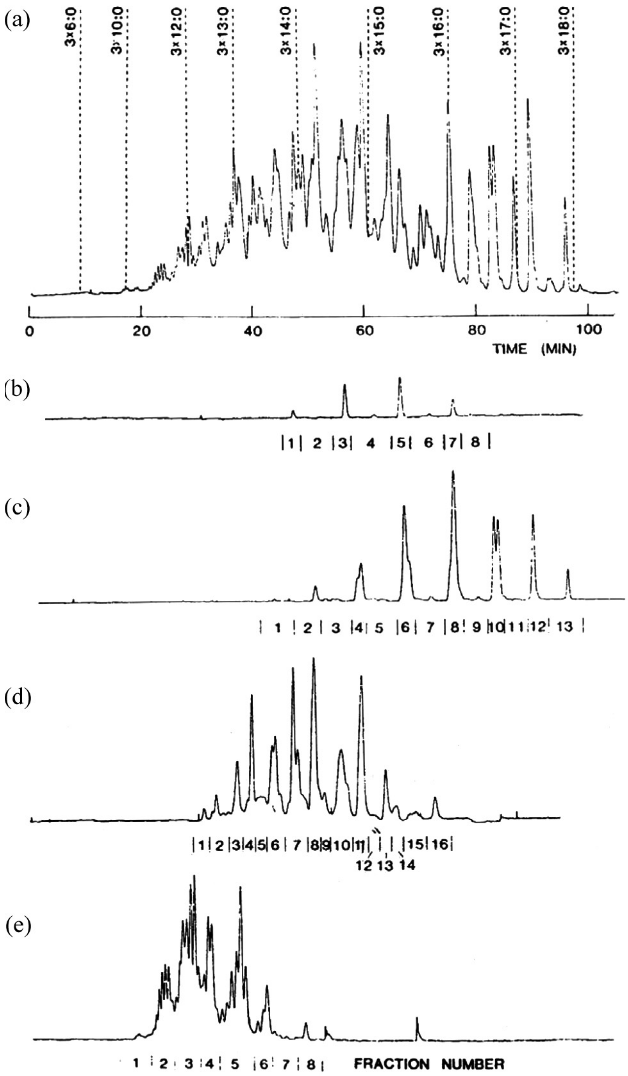


Figure 1. (a) Separation of the total TAGs from herring oil by RP-HPLC with mass detection. Dotted lines show the places where saturated monoacid TAG standards would elute. (b)-(e) RP separation of silver ion HPLC fractions 1, 3, 8 and 11, respectively. The chromatograms are placed so that the peaks are approximately in their correct positions when compared to the total chromatogram. (Reprinted from [38] with permission from AOCS Press).

TAGs profile in analyzed samples in terms of the determination of main compounds as well as minor TAGs, enabling also a good separation of molecules with *cis-trans* differences.

In most of above-described off-line LC-LC separations, practically all fractions of the first column effluent were analyzed also in the second dimension.

Separation of Biological Macromolecules

Most bioactive substances already identified in foods are peptides arising from protein hydrolysis during either processing or *in vivo* digestion. Protein analysis by HPLC is treated in depth by several monographs.[42–44] The HPLC variants most frequently applied are ion exchange (IEC), reversed phase (RPC), hydrophobic interaction (HIC), size-exclusion (SEC) and the various facets of affinity chromatography (AC). Common to all variants, is the feature that they are operated under gradient elution conditions. The supports comprise macroporous crosslinked hydrophilic polymer and chemically bonded silicas. Mobile phases contain buffers, organic solvents, and ion-pairing reagents. The most common detectors employed in protein and peptide analyses are represented by UV absorbance,[45–51] laser-induced fluorescence (LIF),[13] and MS detection.[47,50–58]

Protein hydrolysates are a good example of a complex sample and, therefore, the data interpretation can become difficult due to the overlap of a large number of compounds. To circumvent this problem, multidimensional LC has been widely used for the purification and separation of peptides and proteins. To apply the individual HPLC variants to a multidimensional system for the analysis of peptides and proteins, the following features have to be considered: selectivity, resolution, peak capacity, load capacity, biorecovery, and speed of separation.

Milk and its derivatives have been identified as one of the major sources of biologically active peptides.[59–63] Among others, antibacterial peptides from food protein deserve attention due to their mechanism of activity, which makes microbial resistance improbable,[64] and for increasing the functional values of foods.

Rizzello et al.[65] and Losito et al.[66] described the use of multiple dimension LC for the identification of peptides in antimicrobial fractions of several cheese samples. In these works, a reversed phase fast protein liquid chromatography (RP-FPLC) was used for the fractionation of aqueous extracts of cheese samples. RP LC enables to differentiate proteins according to their hydrophobicities. Generally, a linear gradient is run with increasing content of organic solvents in weakly acid to neutral aqueous/organic mobile phases. The drawback of RP LC is that proteins are often subjected to mobile phase- and stationary phase-induced conformational changes under these conditions. Then, the antibacterial activity of each fraction was assayed toward Lactobacillus sakei A15. The fractions with the highest antimicrobial activity were subjected either to the RP-FPLC separation using a Resource RP column[65] or RP-HPLC with Supelcosil LC-18-DB column[66] coupled to electrospray ionization sequential mass spectrometry (ESI-ITMS^n) with n up to 2[65] or 3[66] and the peptide sequences were identified by comparison with a proteomic database.

The described MS/MS- and MS^3-based method proved to be very successful, leading to the identification of different peptide sequences in fractions of cheese extracts displaying antimicrobial activity. These studies show that several Italian cheeses contain peptides with high levels of homology with well-known antimicrobial or multifunctional peptides. All the peptides were found to be generated by the hydrolysis of milk caseins, which typically occurred in specific regions of the proteins, already known for the presence of bioactive amino acid sequences. Comparison of different cheeses showed that long ripening and intense proteolysis may cause the breakdown of biologically active sequences and that cheeses manufactured with different types of milk (cow, sheep, and goat) have the potential to generate similar peptides with antimicrobial activity. Finally, it was found that chymosin may be one of the major agents responsible for the generation of antimicrobial fragments during cheese ripening (not excluding the potential role of lactic acid bacteria proteases).[65]

The study on an antibacterial peptide using MD HPLC for sample fractionation is described also by Malkoski et al.[67] They studied the caseinomacropeptide (CMP), which is a heterogeneous C-terminal fragment of bovine milk κ-casein (one of the four major types of caseins in milk), composed of glycosylated and phosphorylated forms of different genetic variants. In this study, the growth-inhibitory activity of CMP against the oral opportunistic pathogens *Streptococcus mutans* and *Porphyromonas gingivalis* and against *Escherichia coli* was tested. As a first step, sample preparation consisting of hydrolysis and separation with a TSK gel filtration column connected to an HPLC system was used. Fractions were collected and subjected to MS and N-terminal sequence analysis. The fraction containing CMP was further fractionated by preparative RP-HPLC (with C_{18} column), and fractions collected were characterized by matrix-assisted laser-desorption time-of-flight (MALDI-TOF) MS (equipped with delayed extraction) and N-terminal sequence analysis and then tested for antibacterial activity (for description of antibacterial growth assay see ref.[67]). The active form of CMP was shown to be the nonglycosylated, phosphorylated κ-casein (residues 106 to 169) [k-casein(106–169)], which was designated kappacin. The evidence of

CMP activity against the *Streptococcus mutans, Porphyromonas gingivalis*, and *Escherichia coli* was presented.

Pre-fractionation of the sample using off-line LC-LC has been used for structural analysis of antihypertensive peptides derived from cheese whey[68] and ripened cheeses.[69] Bioactive peptides showing opiate, immunomodulating, antihypertensive, or enhancing mineral-utilization effects have been derived mainly from milk proteins and have been intensively studied.[62,63] Such studies for improving the knowledge on biologically active properties of food are of particular interest, considering that these products are already available on the market and known as functional food.

In the method described by Abubakar et al.[68] and Saito et al.,[69] the digested protein-containing samples were subjected to preliminary fractionation by hydrophobic chromatography for isolation of the peptides, which were subsequently assayed for inhibitory activity of angiotensin-converting enzyme and for changes in the systolic blood pressure caused in spontaneously hypertensive rats after gastric intubation (for determination of the peptides with the strongest depressive effect on the systolic blood pressure). Then, the selected bioactive peptides were chromatographically isolated by combining reversed phase and gel permeation (GPC) modes with UV detection. After the purity of individual peaks was checked with capillary electrophoresis (CE), each peptide was subjected to amino acid sequence analysis by using the automated Edman degradation method (for details see refs.[68,69]). Further, Saito et al.[69] analyzed also the molecular masses of purified peptides by fast atom bombardment (FAB) mass spectrometry.

Bovine whey proteins have potential applications in veterinary medicine, the food industry, and as supplements for cell culture media. Ion exchange chromatography is highly suited for the fractionation of the sample due to its high loadability and high biorecovery. The selectivity of IEC is mainly based on the charge and charge distribution of proteins and on their hydrophobic/hydrophilic interactions when working at high salt concentrations. In order to cover the whole pI range, cation as well as anion exchangers are needed with the appropriate mobile phases. A cation-exchanger-based fractionation scheme for the economically interesting proteins, such as IgG, lactoferrin, and lactoperoxidase, was investigated by Hahn et al.[70] They compared four different cation-exchange media (S-HyperD-F, S-Sepharose FF, Fractogel EMD SO 650 (S) and Macro-Prep High S Support) in regard to their dynamic binding capacity for IgG and their different elution behaviours when sequential step gradients with NaCl buffers were applied. As the first pre-separation step, they used FPLC, and then the collected fractions were analyzed by size-exclusion chromatography and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. Lactoperoxidase activity was monitored by the oxidation of o-phenylenediamine.

This study demonstrates that, due to the high throughput, the selectivity and the simple buffers, the investigated cation exchangers are suitable for large scale fractionation of bovine whey proteins. Depending on the desired purpose, one of the tested materials can be chosen and, if high purity of a certain protein is required, a second purification step must be added.

As can be seen also from previous examples, off-line LC-LC is commonly used as a sample-purification process, followed by further analysis using CE or various MS techniques. One of such example is a work reported by Altmann et al.[71] who analyzed the peptide-N4-(N-acetyl-β-glucosaminyl)asparagine amidase A and its N-glycans in almonds. The N-linked oligosaccharides were released with active PNGase and analyzed as their 2-aminopyridine derivatives by two-dimensional HPLC consisting of SEC in the first and RP-HPLC in the second chromatographic step, followed by MALDI-MS analysis.

A further example, presented by Leitner et al.[72], shows the application of MD HPLC coupled with tandem mass spectrometry for the determination of adulteration of meat products with soybean proteins, which are often added to a variety of food products due to their interesting functional properties and low cost. Their addition to meat products has the scope to increase the emulsion power of meat proteins.[73] Their determination is of particular interest; there are only a limited number of methods available for the (semi)quantitative determination of soybean proteins in processed meat products, but appropriate analytical techniques are needed for the reliable detection or/and quantitation of these additives in meat products due to the fact that regulatory authorities in some countries have forbidden soy protein or limited the amount that can be added in food.[72,74] In the MD LC method described by Leitner et al.,[72] a perfusion LC, developed by Castro-Rubio et al.,[74] was used as a first chromatographic step for the isolation of some fractions from the extracts of soybean protein isolate (SPI) and of meat products containing SPI. The fractions of interest were collected and after enzymatic digestion using trypsin, analyzed by nanoflow LC–MS/MS. In that step, samples were first injected onto a RP trapping column, which was then switched on-line with the analytical RP column. The analytical column was directly connected to a mass spectrometer through an emitter for nanoelectrospray ionization. MS and MS/MS data were processed with the software provided with the instrument and exported in a Mascot-compatible format for identification. The suitability of the method to fulfill the goal was shown; the authors suppose that the presented approach will be also applicable to other food matrices.

Proteolytic reactions play an important role in the development of flavor in protein-rich foods such as cheese, meat, sausage, and fermented soy products. However, knowledge about taste compounds in hydrolyzed plant proteins is limited. Schlichtherle-Cerny and Amadò[75] investigated the chemical composition and the sensory qualities of enzymatic wheat gluten hydrolysates. They employed the taste activity concept as a combination of instrumental and sensory (for details see ref. [75]) analysis to elucidate the contribution of the constituents to the flavor of the hydrolysates, particularly their impact on the "umami" taste (the Japanese word "umami" means delicious and is used as a synonym for the characteristic sensory properties of monosodium glutamate (MSG) and certain purine-5́-nucleotides such as inosine-5́-monophosphate (5́-IMP) or guanosine-5́-monophosphate (5́-GMP)[75]). The wheat gluten was subjected to enzymatic hydrolysis; the obtained hydrolysate was fractionated into 7 fractions by gel permeation chromatography (corresponding chromatogram is illustrated in Figure 2a) using a FPLC system. One taste-active fraction was subfractionated by RP-HPLC (Figure 2b) and further characterization was focused on one fraction, which had a pronounced glutamate-like taste. This fraction was rechromatographed using RP-HPLC (Figure 2c). The effluents corresponding to single peptide peaks were collected, subjected to acidic hydrolysis and analyzed by ESI-MS/MS for detecting their amino acid compositions. The N-terminal amino acid sequence analysis was also performed.

Application of off-line MD LC for the elucidation of the primary structures of the N-linked oligosaccharides from tomato fruit (*Lycopersicon esculentum*) has been described by Zeleny et al.[76] The elucidation of N-glycans in plants is of great interest due to several physiological roles: influence to the correct folding, biological activity and stability of proteins[77,78] and plant development.[79] In this study, the protein fraction was primarily isolated by employing, alternatively, a low temperature acetone powder method and ammonium sulphate precipitation of the tomato extract. After peptic digestion, the glycopeptides were purified by cation-exchange chromatography, which eliminated most of the by-products in the sample. The oligosaccharides were released by N-glycosidase A, separated from peptides and residual glycopeptides by RP LC, and derivatized with fluorescent tag 2-aminopyridine. Structural characterization was accomplished by means of two-dimensional HPLC: the pyridylaminated oligosaccharides were first fractionated by SEC into 11 fractions and these individual fractions were subjected to RP-HPLC, yielding a total of 16 subfractions. LC separations with SEC and RP columns are illustrated in Figure 3. The elution position of each isolated oligosaccharide was determined on both columns and compared to those from reference oligosaccharides. The individual peaks were then subjected to exoglycosidase treatment and the digests were

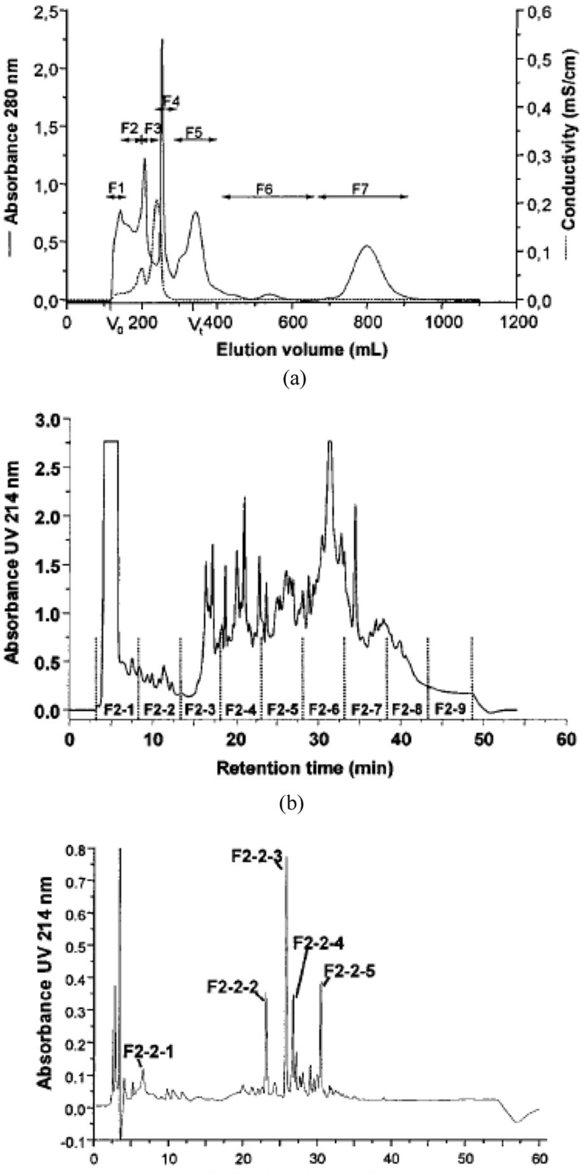


Figure 2. (a) Gel permeation chromatogram of wheat gluten hydrolysate WGH-3 (Mr < 3000; 2.0 g). Vo and Vt are the void and the total volume, respectively, of the packed gel bed. (b) RP-HPLC chromatogram of the semipreparative subfractionation of the taste-active fraction F2. (c) Rechromatography of HPLC subfraction F2-2. (Reprinted from [75] with permission from ACS).

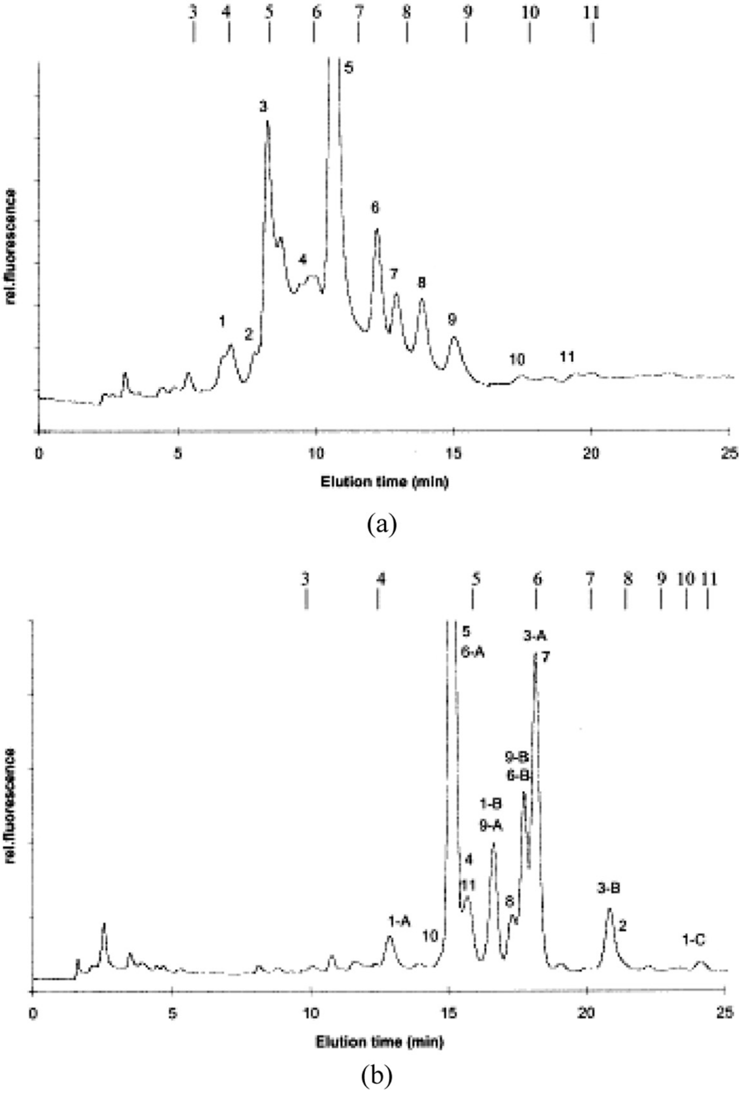


Figure 3. HPLC profles of pyridylaminated oligosaccharides from tomato fruit. The oligosaccharides released with N-glucosidase A were derivatized with 2-aminopyridine and fractionated on MicroPak AX-5 (a) and on Hypersil ODS (b). The individual fractions are indicated by numbers in the charts. Numbers at the top indicate the elution positions of isomaltooligosaccharides with the respective degree of polymerization. (Reprinted from [76] with permission from Elsevier).

again chromatographed on RP and SEC to monitor the change in the elution behaviour, thus yielding even to 3- and 4-dimensional separation. Whenever possible, a tentatively identified component was coinjected with the corresponding reference oligosaccharide to verify the identical elution behaviour. The chromatographic separation was combined with MALDI-TOF mass spectrometry.

In the described study, two varieties of tomato fruit as well as two stages of ripening were investigated. In all samples, the same sixteen N-glycosidic structures were detected; the two most abundant glycans showed identical properties to those of the major N-linked oligosaccharides of horseradish peroxidase and pineapple stem bromelain, respectively, and accounted for about 65–78% of the total glycan amount; oligomannosidic glycans occurred only in small quantities (3-9%). The majority of the N-glycans were β1,2-xylosylated and carried an α1,3-fucose residue linked to the terminal N-acetylglucosamine.

Similarly to the previous study, the next example describes the analysis of N-glycans. In particular, the application of MD LC as an additional investigation to MALDI-TOF-MS analysis of Asn-linked glycans from vegetable foodstuffs is presented by Wilson et al.[80] These kinds of studies are matters of importance for understanding the food allergies. It has to be noted that food glycans are of interest due to the IgE cross-reactions between pollen and vegetable foodstuffs are at least partly due to carbohydrate components[81,82] and it has even been claimed that major food allergens are typically water-soluble glycoproteins.[83]

Wilson et al.[80] studied the structural basis for carbohydrate-mediated IgE cross-reactivity, examining for this purpose 27 food extracts. The investigation of N-glycans was carried out by means of MALDI-TOF-MS. In addition, chromatographic analysis was performed using MD HPLC, which consisted of prefractionation by SEC, followed by secondary fractionation using RP-HPLC. After LC-LC, various exoglycosidase treatments (for details see ref.[80]) and ^{1}H-nuclear magnetic resonance spectroscopy were exploited.

Other Applications

Polycyclic aromatic hydrocarbons (PAHs) represent a heterogeneous class of carcinogens formed by pyrolysis of organic matter. Because of the strong lipophilic characteristic of PAHs, vegetable oils and fatty foods can be heavily contaminated therewith. Most of the methods for PAH determination in oil or fatty extracts involve an extraction step (liquid-liquid partition, saponification, or caffeine complexation) followed by purification through one or more packed columns (silica, alumina, Sephadex LH-20, Florisil) and analytical determination.

An LC-LC method for assessment of polycyclic aromatic hydrocarbon content was developed by Moret and Conte and applied for the analysis of edible oils[84] and lipidic extracts of food.[85] In this method, the PAHs were first pre-separated using a system equipped with a valve and chromatographic column as following: after injection, the sample passed through the backflush valve into the silica column, which isolated the polyaromatic fraction from TAGs. As soon as the fraction of interest had been eluted, the silica column was backflushed in order to clean the column. Next, the PAHs fraction was collected, evaporated to dryness, dissolved in appropriate solvent, and analyzed by RP-HPLC. Regarding detection, UV-Vis and and spectrofluorometric detectors were used. The sensitivity of the described method appears to be 0.1 ppb of a given PAH (injecting at least 100 mg of fat) and the recovery (REC%, tested with PAH concentrations from 4 to 40 ppb) ranged from 37.6 to 102.1% with most of REC% values being around 96% for edible oils[84] and REC% from 63 to 103 with most of REC% values being higher than 95% for fish samples.[85] Moreover, the application of this method for monitoring the PAH content in vacuum-packed trout samples during storage is demonstrated.[85]

Another application of the off-line multidimensional HPLC is the measurement of vitamin D_3 and 25-hydroxyvitamin D_3 (25OHD3) in meat, in which there has been a renewed interest with the increased appreciation of the importance of vitamin D to health, together with improved measurement methods.

The primary dietary supply of vitamin D in human nutrition comes from vitamin D_3 unless supplements or fortification with vitamin D_2 are used. Vitamin D_3 (cholecalciferol) and its active metabolites, 25-hydroxyvitamin D_3 (calcidiol) and 1,25-dihydroxyvitamin D_3 (calcitriol), have been known to be important for calcium and phosphate homeostasis and for the proper development and maintenance of bone, as well as becoming clear that this vitamin also has favorable effects in a number of other health-related areas. Vitamin D homeostasis in humans is complex, involving endogenous biosynthesis and dietary absorption, biotransformation to more active metabolites, and enterohepatic circulation.

Consequently, it is essential to be able to determine at least vitamin D_3 and 25OHD3. Other vitamin D metabolites may also have vitamin D activity, e.g., 1,25-; 24,25- and 25,26-dihydroxyvitamin D, but they are only found in trace amounts in foods.

Jakobsen et al.[86] described a method for the determination of the total vitamin D content in meat. The determination was performed by reversed phase HPLC (using C_{18} columns) after alkaline hydrolysis, solid-phase extraction (SPE), and semi-preparative HPLC with silica and amino columns in series. The fractions containing vitamin D_3 and

25OHD3 were collected and analyzed in RP-HPLC. For detection, a diode array detector (DAD) between 220 and 320 nm was used and quantification was performed at 265 nm. In previous studies it has been shown that the use of an internal standard for the determination of vitamin D is essential, because vitamin D_3, as well as vitamin D_2, show a reversible isomerization with the corresponding previtamin D;[87] the distribution at equilibrium only depends on temperature for vitamin D.[87,88] Hence, also in the work of Jakobsen et al.,[86] vitamin D_2 was used as internal standard both for vitamin D_3 and for 25OHD3. They detected the vitamin D_3 content using two types of saponification for sample preparation: the first at 75°C for 45 min and the second at 20°C for 18 h, obtaining similar quantitative results for both techniques. The described method is applicable for establishing data for food composition tables, in which the information regarding to vitamin D content in meat is rather scarce.

A similar method was used by Purchas et al. for assessment of the vitamin D_3 content in lamb and beef meat ($4+4$ samples) before and after cooking.[89] They also used the procedures of saponification, extraction, and pre-separation (with NP silica column); the fraction containing vitamin D_3 was further analyzed by RP-HPLC and quantified using vitamin D_2 as an internal standard. The assessment of 25-hydroxyvitamin D_3 levels in this work was carried out using a radioimmunoassay method developed for serum.

Some authors have described the use of two liquid chromatographic separations for sample purification and isolation of desired components for further analysis, e.g., with mass spectrometry. For example, McSheehy et al. investigated the selenium content in garlic harvested in naturally seleniferous soil.[90] The absorption, tissue distribution, and body retention of selenium is dependent on the chemical species of the element present in food and food supplements.[91] For this reason, speciation of selenium has been a hot topic in analytical chemistry of natural products. In the method used by McSheehy et al.,[90] the sample was first leached with water, the aqueous extract was fractionated by preparative size-exclusion chromatography and the selenium-containing fraction was analyzed by RP-HPLC with inductively coupled plasma mass spectrometric (ICP-MS) detection in order to verify its chromatographic purity. The garlic extract purified by size-exclusion and reversed phase chromatography was further subjected to electrospray tandem mass spectrometric analysis. The resulting spectrum showed one main ion-cluster corresponding to the four Se isotopes (^{76}Se, ^{77}Se, ^{78}Se, ^{80}Se). This work demonstrated that, in the case of garlic, the use of orthogonal size-exclusion (to isolate amino acid fraction) and reversed phase (to remove salts) chromatographic mechanisms are effective to purify the γ-glutamyl-Se-methylselenocysteine for the characterization by MS/MS. This work demonstrates that tandem MS is an attractive technique allowing the identification of selenocompounds in natural products without standard compounds available. The knowledge of fragmentation patterns may allow, in the future, the use of multiple reaction monitoring mode of very high selectivity and sensitivity for routine screening in HPLC of less purified or unpurified samples.

A similar approach, using three LC dimensions with parallel ICP-MS and ESI-MS/MS detection, was used for the characterization of arsenic species in algae.[92] This multidimensional LC approach included sample cleanup by SEC, isolation of arsenic species by anion-exchange LC, verification of the chromatographic purity of arsenic-containing fractions, and their further purification, if necessary, by RP-HPLC. As the chromatographic detector, an ICP-MS was used and its complementarity with ESI-MS/MS, employed for the identification of the observed peaks was demonstrated. As a result, the following species were found: arsenosugar A $11.7 \pm 0.5\,\mu g\,g^{-1}$, AsV $10.9 \pm 2.1\,\mu g\,g^{-1}$, arsenosugar B $2.22 \pm 0.07\,\mu g\,g^{-1}$, arsenosugar D $1.5 \pm 1.2\,\mu g\,g^{-1}$, a newly detected arsenosugar $1.13 \pm 0.07\,\mu g\,g^{-1}$, arsenosugar C $0.61 \pm 0.04\,\mu g\,g^{-1}$, dimethylarsinate (DMA) $0.42 \pm 0.02\,\mu g\,g^{-1}$ and these accounted for $>99\%$ of the arsenic present. The identities of all the species, except the newly detected compounds, were doubly checked by matching the retention times of chromatographically pure (after the 3rd LC dimension) species with standard compounds and by ESI-MS/MS.

A two-dimensional LC-ICP-MS method, applied to the investigation of the recovery of selenomethionine (Se-Met) from selenized yeast was presented by Polatajko et al.[93] Executed studies using one-dimensional LC showed that recovery of the extracted selenium from an anion-exchange column was 100%, but Se-Met quantified by the method of standard additions accounted only for 67% of the selenium injected. Analysis by SEC/ICP-MS of the eluate collected before and after the Se-Met peak showed the presence of oxidized Se-Met (approx. 3%) and Se-Met likely to be unspecifically associated with the biological matrix continuum (approx.11%). This finding was validated by LC-LC/ICP-MS using two HPLC modes with different elution mechanisms (i.e., size-exclusion and anion-exchange). The developed approach demonstrated that more than 80% of selenium in the selenized yeast is actually present in the form of Se-Met and suggests that many results reported elsewhere for the concentration of this amino acid in selenized yeast may be negatively biased. The research also provided insight into speciation of selenium in the solid residue after proteolytic extraction, but the additional amount of Se-Met recovered was negligible (<1.5%).

Another example, where the two-dimensional liquid chromatography has been used for the purification of the sample, is the work of Lindemann and Hintelmann,[94] who developed an approach for the identification of unknown selenium-containing biomolecules (Selenium-containing glutathione S-conjugates in particular) in a yeast extract. Similarly, with previously described studies, two-dimensional LC was coupled to ICP-MS and complementarily to a hybrid quadrupole TOF-MS/MS equipped with a Z-spray nanoelectrospray ion source. Analytes were first separated by SEC, followed by preconcentration and separation on a porous graphitic carbon HPLC column. The HPLC effluent was monitored for selenium content by ICP-MS and the two selenium-containing fractions were isolated and analyzed by nanoelectrospray MS/MS. This work demonstrates, once more, that the use of HPLC/ICP-MS in combination with MS/MS is an attractive arrangement to identify selenium species for which no authentic standards are available. Nanoelectrospray MS appears to be a promising technique for the acquisition of mass spectra, when only small sample volumes are available. This allows an effective preconcentration of the analyte, which results in a substantial increase in the sensitivity of the method. A two-dimensional chromatographic separation reduced matrix and noise interferences during MS detection. The use of a hybrid quadrupole TOF-MS enabled data acquisition over a broad mass range without significant loss of sensitivity and allowed the acquisition of MS/MS spectra by collision-induced dissociation. The high mass resolution and accurate determination of parent and daughter ion masses allowed a comparison with theoretical masses, which provided further confirmation when identifying new compounds. With the combination of these techniques, it was possible to ascertain the selenium-containing glutathione S-conjugates in an aqueous yeast extract.

On-Line MD LC for the Assessment of the Components Present in Food Samples

On-line LC-LC methods can be used in profiling mode where the aim is to fractionate and separate nearly all components of the sample matrix, but it has found more widespread use in targeted component analysis in the so-called heart-cutting mode. This, as a consequence of the fact that determination of individual analytes in complex samples is a problem which frequently requires some type of sample clean-up or pre-separation as a prerequisite to the analytical measurement.

From a technical viewpoint, the column switching from one dimension to the other is generally carried out by using multiport switching valves. The isolation of selected fractions is generally performed in two ways: using either back-flush or peak heart-cut trapping. In some applications, the first LC is used with the scope to eliminate some unnecessary components of the sample matrix that may interfere with analytes in the sample. A typical on-line coupled-column system for this kind of analysis is illustrated in Figure 4.

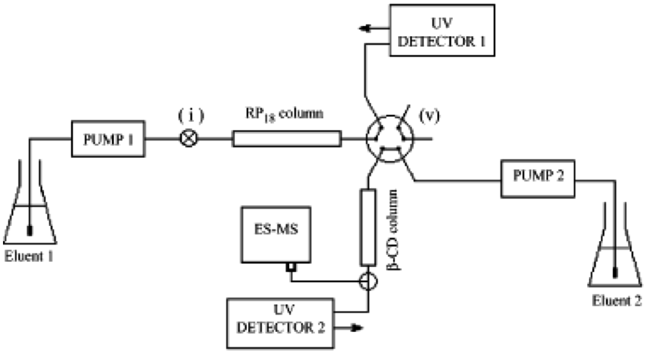


Figure 4. Scheme of the typical coupled column LC: (i) injection valve; (v) six-port valve. (Reprinted from [113] with permission from ACS).

Regarding the applications, on-line MD LC has been used and can be applied to the analysis of various classes of compounds in numerous different food matrixes.

Analysis of Pesticides

A great part of heart-cutting 2D LC techniques concerns the analysis of various pesticides in foodstuffs (e.g., Refs.[95–98]) and water samples (e.g., Refs.[99,100]).

According to the European Community regulations, the maximum amount allowed for single pesticides in drinking water is 0.1 $\mu g\,L^{-1}$ and for the sum of pesticides is 0.5 $\mu g\,L^{-1}$[101,102] therefore, analytical methods with low detection limits (0.02 $\mu g\,L^{-1}$)[103] are required for pesticide monitoring in drinking water.

The current analytical methodology for trace-level determination of pesticides in water usually consists of off-line SPE of water samples and instrumental analysis of the extracts with coupled-column reversed phase LC and UV detection.[104] The main advantage of the use of LC-LC respect to LC is the efficient sample cleanup made by the first RP column, which eliminates the early eluting inferences.[100,104] The use of reversed phase LC is advantageous due to its compatibility with water samples. The solid phase immunosorbents are usually used before the MD LC step with the aim to increase the selectivity of the LC-LC-UV-system, but this step can be avoided by using the high selectivity and sensitivity of the MS detection.

The effectiveness of a coupled-column RP LC method for the analysis of selected herbicides in environmental and drinking water was shown by Hidalgo et al.[99] who developed and applied an LC-LC method for the trace-level analysis of 4 triazine herbicides (simazine, atrazine, terbuthylazine, and terbutryn), which are well known herbicides, extensively applied in agriculture. The RP-RP system used a large volume injection (LVI) of 2 mL, isocratic elution of mixtures of acetonitrile and water in both dimensions, a six-port valve for the column switching, and an ultraviolet detector set at 223 nm. In order to increase the systems sensitivity, a pre-concentration step on C_{18} bonded phase cartridges was added to the LVI-LC-LC system. The developed method is robust, rapid (total analysis time 7 min without pre-concentration step), selective, and sensitive (detection limits yielding around 0.1–0.2 $\mu g\,L^{-1}$ or 0.02–0.05 $\mu g\,L^{-1}$ without and with a preconcentration step, respectively).

The performance of APCI-MS and UV detection in combination, with and without coupled-column RP LC, has been compared for the trace analysis of phenylurea herbicides (used for weed control and public greenery) in various types of water by van der Heeft et al.[100] The comparative study of the on-line LVI-LC-LC/MS (LVI 4 mL of sample) and off-line and on-line LC-LC-UV (with off-line SPE on a C_{18} cartridge) showed that LC-LC-MS is more selective than LC-LC-UV and, in most cases, more sensitive. The LVI-LC-LC/MS approach provided an LOD for most of the analytes down to a level of 0.01 $\mu g\,L^{-1}$. The analysis time of less than 30 min makes it suitable for productive analysis. In regard to LC-LC-UV, the off-line method appeared to be a more viable approach in comparison with the on-line technique. This method allows the screening of phenylurea's in various types of water samples down to a level of at least 0.05 $\mu g\,L^{-1}$. On-line analysis with LVI provided marginal sensitivity (limits of detection of about 0.1 $\mu g\,L^{-1}$) and selectivity was sometimes less in the case of surface water samples. Both the on-line LVI-LC-LC-MS method and the LC-LC-UV method using off-line SPE were validated by analysing a series of real-life reference samples.

Trace-level analysis of targeted compounds is also the main subject of pesticide analysis in foodstuffs of vegetable origin (e.g. Refs.[95,97,98]). The desire to reduce time, cost, and hazardous waste leads to continuous development of alternative means of sample preparation for trace analysis together with the development of automated systems. For selective sample preparation, methods such as SPE[105], supercritical fluid extraction (SFE),[106] and immunoaffinity chromatography (IAC)[107,108] have been used. A combination of on-line sample preparation and analyte confirmation is, for example, presented by G. S. Rule et al.,[97] where the immunoaffinity columns are connected on-line with ion trap (IT) mass spectrometer for the detection of carbofuran (insecticide) in crude potato extract and environmental water samples. In addition, the selectivity of the antibodies for analyte isolation in comparison with ordinary RP column was evaluated. The results showed that the used technique allows one to obtain a considerable degree of sample clean-up and detection of the presence of carbofuran in potato at a level of 2.5 ng g^{-1} (estimated limit of detection). Moreover, with addition of the APCI-IT-MS, full-scan spectra may be obtained to provide confirmation of target analytes with minimal sample preparation.

Combination of LC-LC and MS for pesticide analysis is an effective tool, which, however, presents one drawback while applied to the analysis of complex samples: it is susceptible to matrix-related signal suppression effects,[109–111] which may result in difficulties in qualitative and quantitative characterization of complex samples. The problem was studied by Pascoe et al.[98] They demonstrated that matrix-related signal suppression can be induced either by column overload, by matrix component-analyte coelution, or by the combination of both. Their on-line system consisted of a binary and quaternary pump with the two columns [C_{18}, Restricted Access Media (RAM) or C_8 as the first and C_{18} as the second column] connected using a six-port switching valve and an ESI-MS detection and was applied for the determination of fenozide-herbicides spiked in different matrices (e.g., wheat forage and pecan nut matrix). The absolute recoveries obtained were, on average, greater than the desired value of at least 70%, therefore confirming that the LC-LC/MS(MS) method is effective for the simultaneous quantification of multiple compounds (i.e., up to three per injection) in a complex sample matrix with minimal sample cleanup. Moreover, it was also demonstrated that 2D-LC/MS single-compound analysis (i.e., one compound per injection) could be more effective than multiple-compound analysis in reducing matrix-related signal suppression. The study of single- and multiple-compound analysis confirmed that column leaching from prior eluting compounds could augment signal suppression effects of later eluting analytes. Recovery for the latest eluted compound was increased by an additional 24% for single-compound 2D-LC/MS(MS) analysis versus multiple-compound analyses.

While the works described previously apply effective sample clean-up, an example without this procedure for the trace-level determination of herbicides is reported by Tuinstra and Kienhuis.[95] They described a procedure for glyphosate (very polar non-selective herbicide, which has wide application both post-harvest and even up to 7 days before harvest). In this approach, the vegetable extract in water is injected at first onto an AX/Corasil Bondapack (quarternary ammonium group attached to silica) precolumn. After washing with water, the retained glyphosate is eluted on an Ionosphere A anion-exchange column (quarternary ammonium group attached to silica) with pH 2.1 phosphate buffer eluent, and the eluate is oxidized with hypochlorite and derivatized with o-phthalaldehyde-2-mercaptoethanol reagent for fluorometric detection (excitation 360 nm, emission 400 nm). The precolumn is backflushed and regenerated with 1 M acetic acid. The method was shown to be applicable for the determination of glyphosate in various agricultural products, such as potatoes, strawberries, asparagus, and cereals (barley, wheat, and rye) with a detection limit of 0.2 mg kg^{-1}. Recovery experiments with cereals (tolerance 5–20 mg/kg) at 1 mg kg^{-1} level showed recoveries of 80%. For potatoes, strawberries, and asparagus the tolerance is 0.05 mg kg^{-1}. The experiments at 1 mg/kg level indicated promising results (recoveries about 70%) suitable for working at residue levels, but further improvement of the system (either better pre-column with higher capacity or detection system with higher sensitivity) is necessary for exact quantitation of the glyphosate at the level of 0.05 mg kg^{-1} (described systems' extrapolated detection limit was 0.04 mg kg^{-1}).

Determination of Flavonoid Profile

Two-dimensional LC applied to flavonoid analysis has proved to be suitable both for the evaluation of flavonoid profile[112] as well as for separation of some selected (targeted) compounds.[113] A selective HPLC method with manual column-switching was developed and applied for screening of the flavonoid profiles of white and red berry wines.[112] The system used a C_{18} clean-up column for eliminating the disturbing matrix compounds and a C_{18} analytical column with buffered solvents for separation of flavonoids of interest. A total number of 7 flavonoids (rutin, isoquercitrin, quercitrin, myricetin, quercetin, kaempferol, and flavone, with the last one added as and internal standard) with LOD from 20 to 60 ng mL^{-1} was separated and recoveries from 46 to 70% (to peak heights) and from 76 to 98% (to peak areas) were obtained for samples which were injected without preliminary sample treatment (except filtering) procedure.

An example of the targeted compound analysis is the work of Aturki et al.,[113] who used a coupled-column LC system with UV and negative ion ESI-MS detection for the analyses of flavanone glycoside diastereoisomers in citrus juices. The separation of flavanone glycosides as isomeric forms has significance, both for obtaining insights into the biosynthesis of polyphenolic compounds, as well as for the characterization of quality and authenticity of citrus products.

In the method of Aturki et al., the RP C_{18} column was used for the separation of flavanone glycosides, each of which was subsequently switched into a carboxymethylated β-cyclodextrin (β-CD)-based column and resolved as the corresponding diastereoisomers. Several citrus juices were analyzed: commercial lemon, orange, and grapefruit juices, as well as hand-squeezed juice from fresh oranges, and white and red grapefruits. Figure 5 illustrates the diastereoisomer separation of hesperidin from orange juice (Figure 5a), naringin and narirutin from grapefruit juice (Figure 5b) and eriocitrin from lemon juice (Figure 5c). Qualitative and

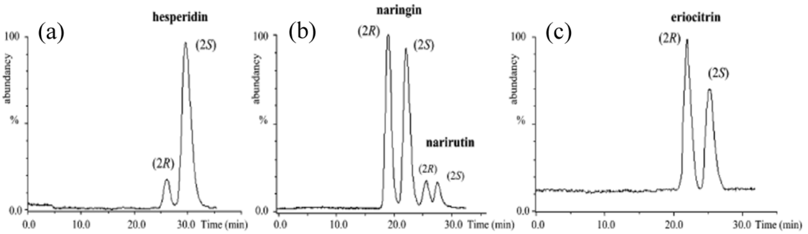


Figure 5. Separation of flavanone glycoside diastereomers in citrus juice extracts on columns packed with ODS/CM-β-CD-bonded stationary phases: (a) orange juice; (b) (red) grapefruit juice; (c) lemon juice. (Reprinted from [113] with permission from ACS).

quantitative determination of diastereoisomers of five flavanone glycosides (eriocitrin, naringin, narirutin, hesperidin, neohesperidin) was carried out. The recovery of these compounds (determined after switching the compounds from one column to the other), calculated for naringin from grapefruit extract, was 86.0 ± 2.8%.

Separation of Peptides and Proteins

When talking about automated or multidimensional HPLC systems, it is impossible not to mention the application of these techniques to the separation of proteins and peptides with no exception in the field of food analysis. Although these kinds of analyses have been generally performed using 2D gel electrophoresis, the growing interest regarding the effective and selective separation/determination/identification of these compounds in complex samples of natural origin leads to the use of on-line LC-LC techniques.

Systems for proteomics studies in tryptic digest of bovine milk have been examined by L. Riggs et al.[114] The described signature peptide approach for the separation of phosphorylated proteins involves at least six steps: reduction, alcylation, proteolysis, affinity selection of signature peptides, reversed phase chromatography, and electrospray MS. Peptides were automatically reduced and alkylated in the autosampler of the instrument, followed by trypsin digestion of all proteins in the sample on an immobilized enzyme column and the direct transfer of the digest to an affinity chromatography column, which was a Ga(III) loaded immobilized metal affinity chromatography (IMAC) column, known to select phosphorylated peptides. The phosphorylated peptides selected by the affinity column were automatically transferred to a RP column, on which the further fractionation of tryptic peptides was achieved with linear gradient elution. The effluent from the RP LC column was directed into an ESI-TOF-MS. The system was equipped with three (trypsin,

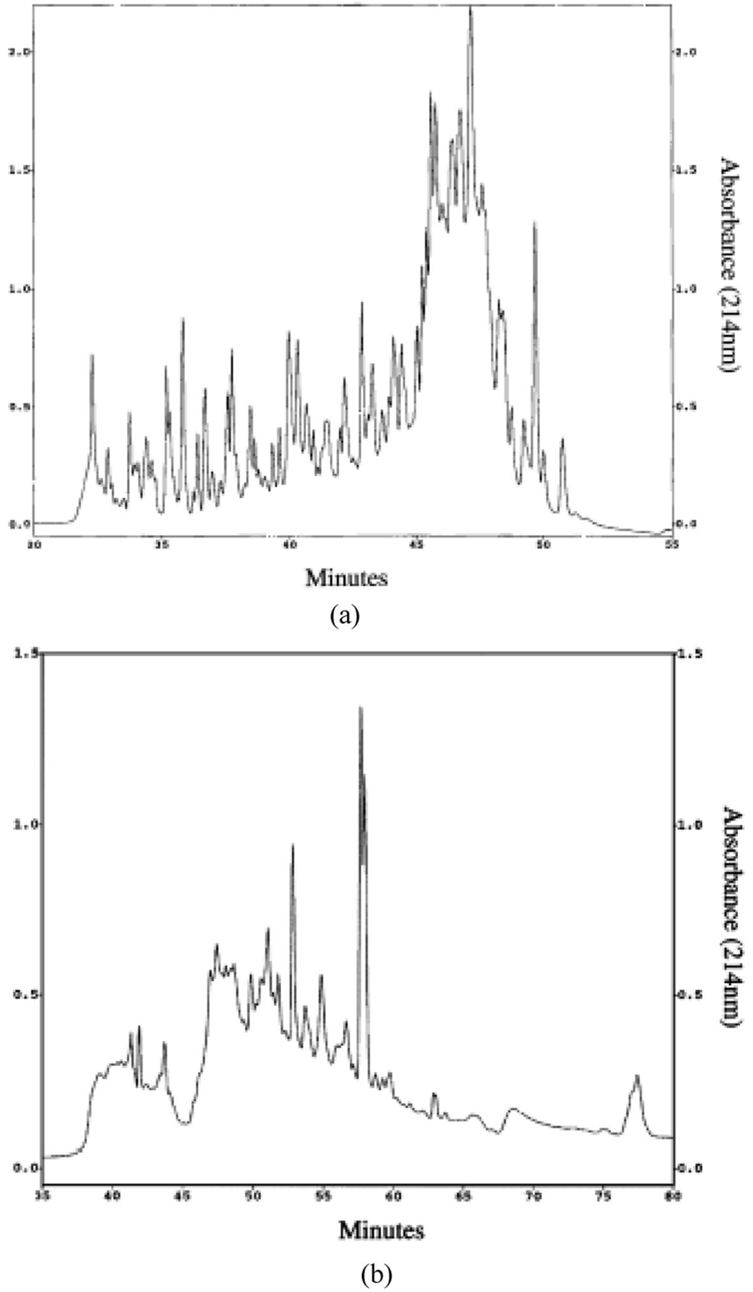


Figure 6. (a) Reversed-phase chromatogram of digested milk resolved on a PepMap C_{18} RP column. Elution was achieved with a linear gradient ranging from 0.1% acetonitrile (ACN) and 0.1% formic acid in double di-ionized water (ddI water) to 95% ACN–0.1% formic acid in ddI water at 1 mL/min over 60 min. (b) Phosphorylated milk peptides Ga(III)–IMAC selected and resolved by RP-LC/MS. (Reprinted from [114] with permission from Elsevier).

IMAC, and RP) columns, connected by means of three 10-port switching valves. Figure 6 enables the comparison of the RP chromatograms of two types of milk analyzed. Figure 6a represents the RP chromatogram of digested skimmed milk. As it is possible to see, at the later stage of the gradient, the chromatogram presents a group of poorly resolved peptides. Based on the known structures of the casein components, it is estimated that over 300 peptides will be produced from a tryptic digest of skimmed milk. Figure 6b shows the RP chromatogram of peptides captured from the same milk digest by the Ga(III)–IMAC column. Peptides released from the IMAC column were directly transferred to the RP column. Approximately 22 peaks were detected from the initial mixture of more than 300 peptides.

The authors notice that with slight modification, it is possible to add multiple columns in parallel at any of the single column positions to further increase throughput. Total analysis time of the automated multistep method was little more than 1.5 h, which is greatly shorter that the time required for the same process performed by solution digest and manual analysis (10 h and 40 min as reported by Riggs et al.[114]).

Single amino acid analysis has been described by Dossena et al.,[115] who described the determination of enantiomeric composition (e.g., D- and L-amino acids, including proline) of complex mixtures of amino acids, such as those occurring in biological fluids and foods. The amino acid separation was achieved using an ion exchange column and elution with a lithium chloride-lithium citrate buffer. Each peak corresponding to an individual amino acid was switched to a reversed phase column (C_{18}) and eluted with an aqueous solution. The enantiomeric separation was achieved by adding to the mobile phase chiral copper(II) complexes, which perform chiral discrimination by a ligand-exchange mechanism. An unnecessary overrunning of the chiral system with the whole mixture was avoided by switching only the peaks under investigation into the secondary column. The results obtained showed the possibility to evaluate D-amino acids up to a 0.1% D to $D + L$ ratio in the nanomolar range. Postcolumn derivatization with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole and fluorometric detection were utilized for proline and hydroxyproline and with o-phthalaldehyde for the other amino acids. The method described is very flexible as several chiral selectors and different types of detection (e.g., UV, fluorescence) can be used.

Several other applications of MD HPLC have been reported, such as for example, targeted MD analysis of malt acrospires,[116] which have an unfavourable influence on beer flavour stability. The astringent substance from acrospires was extracted and analyzed by MD LC.

Thompson and Plouffe[117] described a 2D method for the measurement of cholecalciferol in meats and fats, with the aim to examine the edible tissues from overdosed farm animals. The method consisted of four steps: alcoholic digestion, clean-up on alumina and two-dimensional HPLC, using NP with silica column in the first (^{1}D) and RP column in the second (^{2}D) dimension. The results showed the levels in meat and fat ranging from normal (0.2 μg per 100 g) to excessive (200 μg per 100 g). As an extension of the procedure, the measurement of 25-hydroxycholecalciferol is described, even if residues in the form of this metabolite are concluded to be of lesser practical significance. Regarding the detection, an absorbance detector at 254 nm is recommended for the detection and identification of cholecalciferol.

Another example of a multi-step process, where the sample clean-up, derivatisation, and separation was in a closed on-line system, concerns the determination of biogenic amines in wines.[118] The procedure was as follows: the sample was injected onto the cation-exchange pre-column, where the majority of matrix compounds was removed by washing. After washing, the amines were eluted in back-flush mode to the derivatisation coil with a mixture of the buffer and acetonitrile ($85 + 15$, v/v). Then, the amines were separated on a C_{18} analytical column and detected by a fluorescence detector. As a result, 9 amines were separated and quantified in different red wine samples. The system proved to be highly sensitive, presenting LOD below 0.05 mg L^{-1} for all amines and linearity preserved over the tested range (0.05–15 mg L^{-1}). The sensitivity of the system can be increased even further by increasing the sample size. The described LC-LC system with on-line derivatization, where the only manual pre-treatment was dilution of the sample and filtration, can be easily automated.

A number of on-line LC-GC methods involve two LC steps for sample pre-separation (LC-LC-GC).[119–122] The analysis of mineral oil polyaromatic hydrocarbons in foods is such an application.[122] It has been carried out with an automated on-line method, in which a first LC separation for the removal of fatty part from the sample matrix is performed on a silica column, followed by the evaporation of the solvent from the obtained hydrocarbon fraction in an on-line solvent evaporator (SE) and a second LC separation of hydrocarbons using an aminosilane-derivatized column followed by UV detection. After this, the components of interest were fractionated and transferred to a GC through the in-line vaporiser/overflow interface. Figure 7 illustrates the described LC-SE-LC-UV and -GC-FID separation. Direct LC-LC presupposes, however, that the fraction eluted from the first LC column is focused at the inlet of the second column. This may be achieved by selection of a second LC column with an increased retention power (used for the methods mentioned above) or by online dilution of the eluent with a weak solvent.[123] If such focusing is not feasible, reconcentration requires evaporation of the eluent from the fraction of the ^{1}D column before it is introduced into the ^{2}D column. This is also a case of the above-described method. The method was designed for the injection of a large

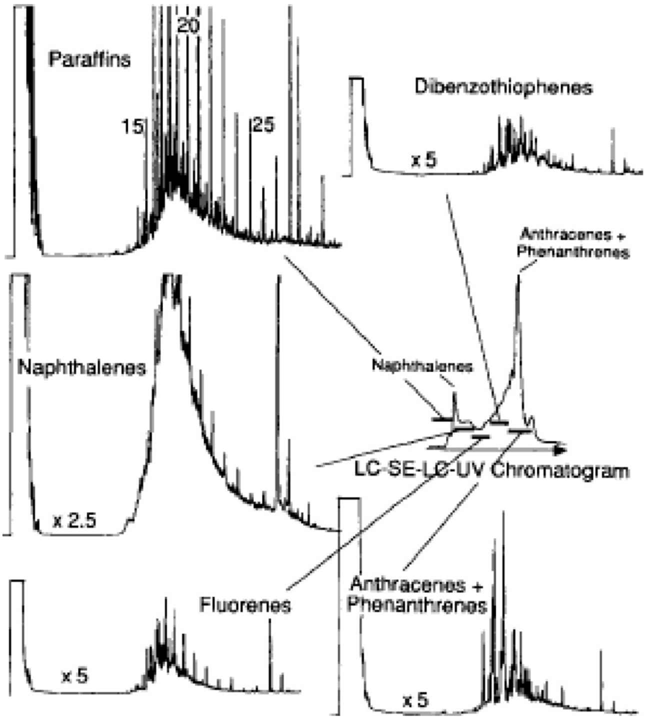


Figure 7. LC-SE-LC-UV and -GC-FID for the analysis of mineral oil material in a non-refined linseed oil. (Reprinted from [122] with permission from Elsevier).

amount of food extract (e.g., up to 200 mg of fat) and was applied to the analysis of mineral oil material in a linseed oil.

Comprehensive HPLC in Food Analysis

Comprehensive HPLC systems, developed and applied to the analysis of food matrices up to now, employed the combination of either NPxRP or RPxRP separation modes. However, at this point, it is important to notice that the displacement of the two separation mechanisms used should be as orthogonal as possible, so that no or little correlation exists between the retention of compounds of both dimensions. In NP mode, two different methods of separation have been explored: adsorption with polar inorganic packing material, generally silica,[124,125] and partition, in which a polar organic phase is chemically bonded to the silica substrate, such as cyanopropyl (CN)[126] or diol groups.[127]

For triacylglycerol separation, AgxRP combination has been used. The Ag-HPLC mode, that uses solvents similar to those used in NP chromatography, has been investigated using a lab-silvered micro-bore cation exchange column.[22,128,129] So, the principles of coupling of Ag and RP modes in comprehensive HPLC are very similar to those of NPxRP HPLC.

In NPxRP set-up, C_{18} is the most commonly employed RP stationary phase,[22,124–129] while for RPxRP set-up, the effect of several stationary phase chemistries (C_{18}, PEG, SCX, CN, NH_2, Phenyl) on the separation selectivity and resolution has been investigated. For separation of antioxidants, phenyl–silica and PEG–silica phases, besides conventional octadecyl-silicas, have received great attention.[130–132] The selectivity of the phenyl–silica column is principally based on π–π electron interactions of the aromatic rings, whereas the PEG-phase selectivity utilizes mainly hydrogen-bonding polar interactions, in addition to hydrophobic (reversed phase) interactions present on both types of columns.

Evidently, when setting up an LC × LC system, both the choice of the columns for the two dimensions as well as of the operational parameters is crucial. This has, up to now, mainly been performed by using trial-and-error strategies. Notable exceptions are the works by Schoenmakers et al., who described a protocol for designing comprehensive two-dimensional HPLC separation systems[133] and Bedani et al., who, in order to make the optimization process less empirical, described a theoretical basis for parameter selection and instrument design for comprehensive 2D system consisting of size exclusion chromatography and liquid chromatography.[134] The main difference between these two works comes from a practical perspective: while, in the former, the complete optimization of the 2D system is described as such without any *a priori* boundary conditions; the starting point in the latter is an existing one-dimensional separation for which the desire is to improve peak capacity without the need to re-optimize the original separation.

LC × LC Chromatography of Triacylglycerols

Various lipidic matrixes such as donkey milk samples[22] and plant oils[128,129,135] have been investigated using a comprehensive 2D LC mode employing silver ion HPLC in one dimension and non-aqueous RP HPLC in the other one. The comprehensive approach was based on the employment of ^{1}D microbore Ag column and of ^{2}D octadecylsilyl column connected via a 10-port switching valve. Nakashima et al.[135] equipped the valve with one sample loop operating at constant pressure with a stopped flow method; this allowed one to change the sampling time (i.e., injection volume) independently of the 2nd separation time. The thorough elucidation of rice-oil TAG profile was reported by Mondello et al. using a three-dimensional Ag LC × RP LC/APCI-MS method.[128] A primary Ag microbore column, operated under isocratic elution conditions

(0.7% v/v acetonitrile in n-hexane) was connected to a monolithic column, operated under gradient elution conditions (various isopropanol and acetonitrile mixtures) at 4 mL/min flow rates, through a 10-port valve with two 20-µL loops. The flow rate in the first dimension was mantained at 13 µL/min, which corresponded to a modulation period of 1.5 min. In this way, 83 fractions of primary-column effluent were subjected to a further rapid separation. Due to the highest LC × LC resolving power and MS spectral information, 11 triacylglycerols were positively identified.

A nice application to the analysis of TAGs in donkey milk, using a similar set-up, has been reported by Dugo et al.[22] With respect to the previous work, the main difference consisted in the use of gradient elution also in the first dimension, enabling the attainment of peaks with comparable widths in a reasonable analytical time. By using this approach nearly 60 TAGs were separated and identified revealing that the investigated system is an effective tool for the characterization of TAG fraction of such a complex matrix.

The use of a gradient program in both LC dimensions was also applied to the analysis of different plant oils containing TAGs in a wide range of PN (36–52) and DB numbers (1-9) such as linseed and soybean.[129] Moreover, also the separation of some positional isomers was achieved.

In all the cases, TAGs were located in specific positions of the 2D chromatograms, in relation to their PN and DB values. Figure 8 illustrates the LC × LC separation of soybean oil TAGs with mentioned

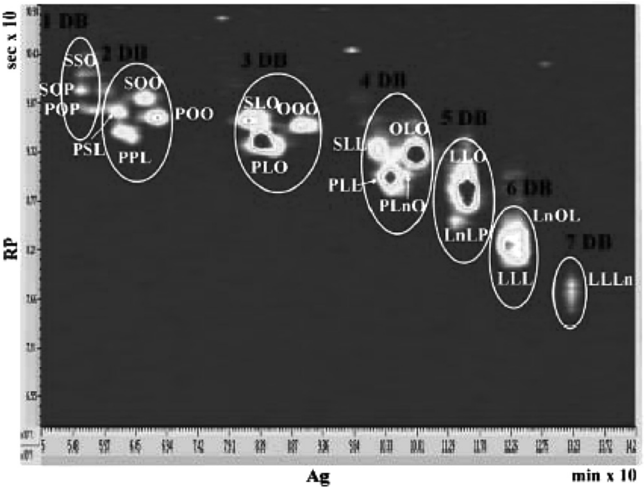


Figure 8. Comprehensive 2D silver ion – reversed phase LC separation (with APCI-MS detection) of the TAG fraction of soybean oil. (Reprinted from [122] with permission from Elsevier).

DB zones indicated. This makes easier peak identification even when commercial standards are not available. The use of APCI-MS greatly increased the potential for component identification thanks to the absence of peak coelution and to the restricted number of components that can be assigned to a spot located in a well-defined position of the 2D plane.

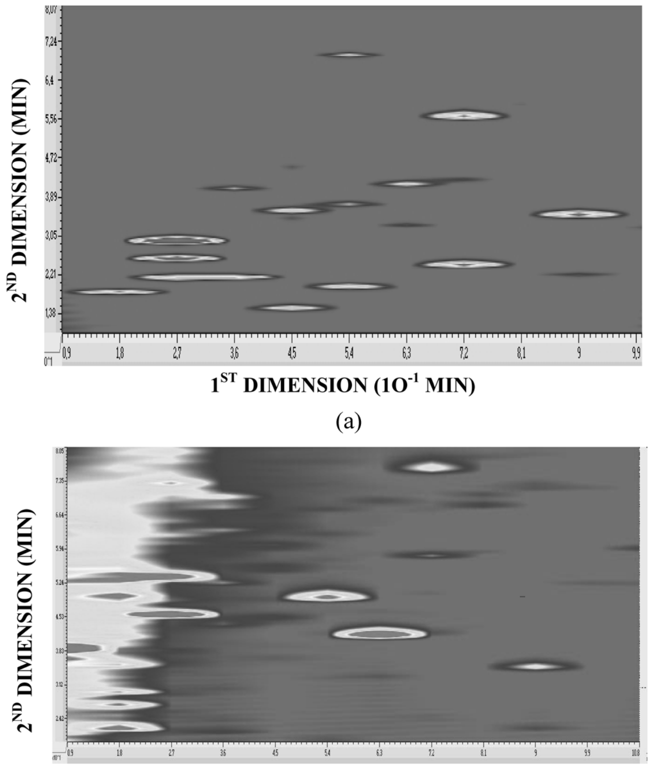
LC × LC Chromatography of Phenolic Antioxidants

Phenolic antioxidants make up a large family of naturally occurring compounds in fruits, vegetables, and beverages originating from the plants used in production such as barley, hop, and hop products used in the beer brewing process. Some of them present strong antioxidant properties with benefical, physiological, and anticarcinogenic properties for human health.[136–138]

HPLC with UV spectrophotometric detection[139–147] represents the most popular and reliable technique for their determination, even if electrochemical detectors have recently received great attention.[139,148–150]

Because of high peak capacity, selectivity, and resolution, 2D LC × LC system have been proposed for the separation of these compounds, taking the advantage of the different selectivity of various RP columns. In most works reported in the literature, various combinations of columns have been employed for the separation of these compounds in herbs, beer, and wine samples. Cacciola et al. explored the use of various combination of PEG-silica, phenyl-silica, and C_{18} columns in a single column or serial (tandem) arrangement in the first dimension and either a monolithic or a ZR-Carbon column in the second dimension.[130–132,151] The combination of different stationary phase chemistries provided low selectivity correlations and high dispersion of retention data between the two dimensions. In particular, the use of a PEG-silica (due to the high polarity of the bonded phase) and a ZR-Carbon column (that allowed the use of high temperatures due to high thermal stability) provided complementary selectivity to C_{18} columns to be employed in the comprehensive 2D system. Moreover, parallel and matching gradients in the two dimensions allowed to improve system orthogonality, bandwiths suppression and more regular band distribution over the whole 2D retention plane.[130] In most cases, instead of sampling loops, either two alternating trapping X-Terra columns for sample fraction transfer from ^{1}D to ^{2}D[130,132] or two analytical ^{2}D columns in parallel[132,151] were used. Figure 9 shows the comprehensive LC separation of a mixture of standards of phenolic antioxidants and flavonoids (A) and of a pilot beer sample (B).

A comprehensive LC system interfaced to ESI-TOF-MS has been employed for analysis and quantification of phenolic acids contained in Lamiaceae herbs.[152] In this study, a dynamic sonication assisted



(a)

(b)

Figure 9. (a) RPxRP separation of a mixture of phenolic antioxidants and flavonoids (for peak identification see ref. Cacciola et al. J. Sep. Sci. 2006 29 2500–2513); (b) RPxRP separation of a mixture of phenolic antioxidants and flavonoids contained in a pilot beer sample. (Reprinted from [132] with permission from Wiley).

extraction method was developed and compared to traditional extraction methods. Selected antioxidative phenolic acids and flavonoids have been also determined from wines and juices by using either an octadecylsilica in the first dimension and a CN column in the second dimension or two C_{18} columns in both dimensions by means of different mobile phase compositions.[153]

An increasing consumption of sweeteners present in food has resulted in several nutritional and medical problems. For this reason, a great interest was devoted to *Stevia Rebaudiana* as alternative to these synthethic compounds for treatment of diabetes, hypertension, and for prevention of caries. Pol et al.[154] developed different combinations of cation-exchange, amino and octadecylsiloxane stationary phases in comprehensive LC mode for the separation of glycosides contained in an aqueous extract of this herb in connection with electrospray ionisation time-of-flight mass spectrometry.

Other Applications

Other food matrices have been investigated by comprehensive NP LCxRP LC. One of the best examples is represented by the analysis of oxygen heterocyclic components in cold-pressed lemon oil, by using normal phase with a microbore silica column in the first dimension and a monolithic C_{18} column in the second dimension with a 10-port switching valve as interface.[124] Oxygen heterocyclic components (coumarins, psoralens polymethoxylated components) represent the main part of the non-volatile fraction of cold-pressed citrus oils. Their structures and substituents have an important role in the characterization of these oils, since their qualitative and quantitative composition is characteristic of each oil. Positive peak identification of these compounds was obtained by both the relative location of the peaks in the 2D plane, which varied in relation to their chemical structures and by characteristic UV spectra that greatly supported the identification of the analyzed components. Later on, a similar set-up was used for a citrus oil extract composed of lemon and orange oil.[127] The main difference with respect to the earlier published work[124] was the employment of a bonded phase (diol) column in the first dimension. Under optimized LC conditions, the high degree of orthogonality between the NP and RP systems tested, resulted in peak capacities of 450.

A novel approach for the analysis of carotenoids, pigments mainly distributed in plant-derived foods, especially in orange and mandarin essential oils, has been recently developed by Dugo et al.[125,126] In terms of structures, food carotenoids are polyene hydrocarbons, characterized by a C_{40} skeleton that derives from eight isoprene units. They present an extended conjugated DB system that is responsible for the yellow, orange, or red colors in plants and are notable for their wide distribution, structural diversity, and various functions. Carotenoids are usually classified into two main groups: hydrocarbon carotenoids, known as carotenes (e.g., β-carotene and lycopene), and oxygenated carotenoids, known as xanthophylls (e.g., β-cryptoxanthin and lutein). The elucidation of carotenoid patterns is particularly challenging, because of the complex composition of carotenoids in natural matrices, their great structural diversity and their extreme instability. An innovative comprehensive dual-gradient elution HPLC was employed in connection with a NP LCxRP LC set-up, which was composed of silica and C_{18} columns in the first and second dimension, respectively. Free carotenoids in orange essential oil and juice (after saponification), were identified by combining the two-dimensional retention data with the UV-visible spectra[125]

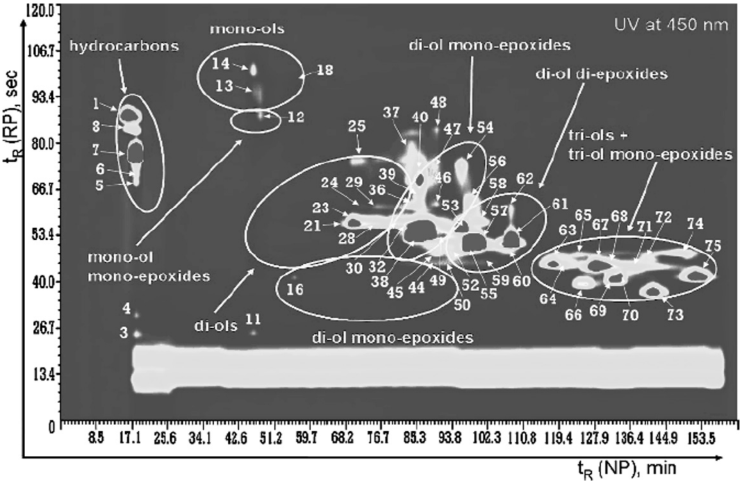


Figure 10. Contour plot of the comprehensive HPLC analyses of carotenoids present in red orange juice with peaks (for the identification see ref. 125) and compound classes indicated. (Reprinted from [125] with permission from ACS)

obtained by PDA detector. Figure 10 shows the comprehensive LC chromatogram of the carotenoid fraction of a saponified red orange juice. In the later study,[126] the ^{1}D microbore silica column was replaced with a cyanopropyl column for the separation of esters. Regarding detection, an employment of the PDA in connection with the APCI-MS provided reliable identificationof free carotenoids and carotenoid esters, as described in the research of mandarin essential oil sample.[126]

2D LC × LC tecniques have also found applications for the separation and characterization of alcohol ethoxylates, nonionic surfactans widely applied as solubilizers in food.[155] The combination of normal phase with aqueous solvents and reversed phase accomplished the simultaneous alkyl and ethylene oxide distribution analysis.

CONCLUSIONS

The information gleaned from the studies of food composition has a great impact on the quality of human life. The demand for reliable, precise, and accurate data in the analysis of food samples is growing increasingly. This is partly related to an increased necessity for a comprehensive characterization (or, in other words, a complete view) of various products due to legislation requirements, health concerns, controlled processing, etc. In order to meet this challenging demand, significant technological evolution is of fundamental importance.

In the field of liquid chromatography, which has undergone extensive improvements and adaptations, some of the greatest and most significant advances are connected to the development and introduction of multidimensional systems, which extend the limits of analytical capabilities of conventional LC methods.

The number of references cited in this review, even if surely representing only a small part of the applications of MD LC in food composition analysis, show that the multidimensional HPLC is having, and will have, an important role along side other separation techniques in this application area.

This review demonstrates that a vast number of LC techniques have been used in MD LC mode for the analysis of a big variety of matrices. Advances in multidimensional liquid chromatography coupled to several detection systems, of which the most powerful is definitely the mass spectrometry, leads to systems capable to afford effective analysis both of the total profile of sample components as well as trace-level determination of less-abundant compounds.

However, the great separative and identificative power (especially in the case of comprehensive systems) of 2D tecniques makes them very attractive in the analysis of various complex samples and the application to food analysis represents the next step toward the simultaneous separation and identification of such products, especially where the availability of information on the composition of the sample as well as of commercial standards is rather limited.

Still limited is the number of applications of comprehensive two-dimensional HPLC to food analysis if compared to conventional off-line and on-line MD LC. This is probably due to the fact that comprehensive 2D LC is a relatively new technique, and that its capabilities in this specific field are still unexplored. Up to now, this technique has been mainly used in the field of polymers[20,156–162] as well as biological samples.[45–58,134,163–165]

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